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Recent advances in understanding the invasion of erythrocytes by merozoites of *Plasmodium knowlesi* *

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Previous observations on the process of invasion by P. knowlesi *are briefly reviewed and new findings concerning the adhesion of parasites to erythrocytes and their intracellular passage are reported. Merozoites adhere to erythrocytes by means of a well-defined coat with the cytochemical characteristics of glycoprotein. This coat has receptors that engage in at least three distinct types of attachment. The ensuing invagination of the erythrocyte surface has two phases, the first consisting of an inward buckling of the membrane to form a vacuole and the second a rapid expansion to create the final parasitophorous vacuole inhabited by the trophic parasite. Some cytochemical evidence concerning the nature of the rhoptry complex is discussed in relation to these changes.*

When a malaria merozoite invades an erythrocyte, it undergoes a complex sequence of activities that begin with its adherence to the host cell and end when, having reached the cell's interior, it transforms itself into a trophic form. These changes can be observed in detail only with the electron microscope because of the small size of the parasite. It was as recently as 1969 that Ladda et al. (*1*) gave the first detailed description of invasion and showed for the first time that the parasite causes invagination of the erythrocyte surface to form a parasitophorous vacuole into which the merozoite moves, and does not penetrate the cell membrane as had been thought previously.

Since this early study on *P. gallinaceum* and *P. berghei*, other investigations have been carried out with *in vitro* preparations of *P. knowlesi* (*2*, *3*), which have confirmed and amplified the findings in the other species. In particular, the development of a method for separating live merozoites from infected rhesus monkey blood by Dennis et al. (*4*) has facilitated research on *P. knowlesi* merozoites and their invasive behaviour. This technique involves the incubation of late-stage schizonts, previously concentrated by centrifugation, in a stirred, gassed, and temperature controlled container, the floor of which is a polycarbonate filter with 2-μm-diameter pores, which are large enough to allow merozoites to pass but sufficiently small to prevent schizonts from doing so. Merozoites prepared in this manner show decreasing viability over a period of 30 min when kept at 4°C. Freshly harvested merozoites can be incubated with erythrocytes at 37°C for invasion studies or otherwise processed for other types of investigation.

In the present paper, we shall summarize the results of such studies on *P. knowlesi* and attempt to correlate them with what is known about invasion by *Plasmodium* spp. from other sources. Before proceeding to a detailed consideration of invasion in its various aspects, we shall briefly outline the chief events of this process.

INVASION: GENERAL FEATURES

Merozoites attach by any part of their surface to erythrocytes, those adhering by their apical prominence passing rapidly to the next stage of invasion and those attaching by other regions causing the erythrocyte to undergo spasmodic distortion before the apical prominence makes contact with the erythrocyte membrane. In electron microscope preparations, the merozoite is visibly attached by means of its cell coat. Next, the parasite induces the erythrocyte membrane to invaginate and subsequently passes into the cavity so formed, leaving its cell coat at the rim of the invagination. Ladda et al. (*1*) suggested that vacuole formation results from the release of membrane-active materials from the

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apical complex organelles (rhoptries and micronemes). We have further suggested that the merozoite is drawn passively into the forming vacuole by fluid movements generated by the inward expansion of the erythrocyte membrane (*2*). The merozoite is then completely surrounded by the vacuole, which becomes sealed off from contact with the exterior; the merozoite subsequently releases other densely staining vesicles (microspheres) through its pellicle, so causing further expansion of the enclosing parasitophorous vacuole. At the same time, the parasite loses its rounded shape, the pellicular membranes are reduced to a single plasma membrane, and the parasite transforms into the next, trophic stage of parasitic life.

The mechanisms involved in these various processes are poorly understood. Recent work in this laboratory has produced more data relevant to this subject, and these will now be discussed.

THE MEROZOITE SURFACE AND ADHESION

The surface of *P. knowlesi* merozoites is covered by a thick cell coat, which is attached to the plasma membrane and usually extends about 20 nm from the membrane surface (*1*, *5*). There has been a measure of uncertainty as to the nature of this coat. Ladda et al. (*1*) first pointed out its existence and its curious shedding that occurs during the entry of the parasite into the erythrocyte. Although there has been some doubt as to whether it is an integral part of the parasite, Miller et al. (*6*) showed that it is associated with parasite antigens and must therefore have arisen from the schizont either as part of the merozoite's intrinsic structure or perhaps as something added in the schizont vacuole and loosely adherent to the parasite's surface. We have found that the cell coat is present in a highly ordered state on the merozoite exterior from an early stage in merozoite development (i.e., as soon as the pellicle of the forming merozoites begins to differentiate at the perimeter of the early schizont) and is visible throughout merogony and after schizont rupture in extracellular merozoites (*2*). The coat has an unusual structure, consisting of regularly spaced T- or Y-shaped bristles mounted perpendicular to the cell membrane (Fig. 1, 2, 3). Each bristle has a thicker (5-nm) stem about 20 nm long and two thinner apical filaments at its distal end; these normally lie parallel to the cell surface to form the outer boundary of the cell coat, but may also extend vertically by as much as 40 nm when adhering to an adjacent cell (see below). In *P. yoelii*, such bristles are arranged in regular rows on the merozoite surface. The cell coat in *P. knowlesi* stains strongly with ethanolic phosphotungstic acid, can be removed with proteases, and binds ruthenium red, alcian blue/lanthanum nitrate, and cationized ferritin (Fig. 4) but not uncharged ferritin. The merozoite also possesses a net negative charge, as determined electrophoretically (*2*). It is therefore likely that the cell coat is an anionic glycoprotein. Recent experiments in this laboratory with various lectins, including phytohaemagglutinin, wheat germ agglutinin, *Ricinus communis* agglutinin, and concanavalin A, have failed to cause agglutination of merozoites, although some concanavalin A–ferritin binding is detectable; this suggests that any glycosides that might bind such lectins are situated within rather than at the external boundary of the cell coat.

In all types of adhesion by merozoites to other cell surfaces, the surface coat of the merozoite is interposed between adjacent cell membranes (*2*, *3*) and there is structural evidence that the considerable forces generated between merozoites and erythrocytes depend on a firm attachment of the parasite by means of this adhesive coat (*2*). These considerations indicate that the cell coat of the merozoite is a structural feature of its plasma membrane that enables it to capture erythrocytes prior to actual invasion.

Adhesive behaviour of merozoites

The adhesion of merozoites to erythrocytes has recently been examined by Miller et al. (*7*) in terms of receptor sites on the erythrocyte surface necessary for the attachment of merozoites. Little is known about the structural basis of such merozoite–erythrocyte interactions. We have examined, by means of transmission and scanning electron microscopy, the types of adhesion in which merozoites engage and have detected evidence for at least three categories of intercellular adhesion, as follows:

Merozoite–merozoite attachment is observed within the schizont and between recently released merozoites. Such merozoites are held together with long, tenuous threads (also observed in schizonts of *P. simium* by Seed et al., *8*), which appear to be the distal filamentous portions of the merozoite coat bristles, extending up to 6 nm between cell surfaces. Attachments of this kind are temporary and are easily ruptured.

Long-distance merozoite–erythrocyte attachments are also mediated by long extensions of the cell

coat bristles and typically occur between nonapical parts of the merozoite and the erythrocyte, which is often distorted into a cup-like form (Fig. 5). Attachments of this kind may span distances of 60 nm or perhaps more, and may be responsible for the initial capture of the red cell.

Short-distance merozoite–erythrocyte attachments are typically found between the apical prominence of the merozoite and the erythrocyte (Fig. 6) and both surfaces may become distorted as though considerable binding forces were present at such contacts. The distance between cell membranes is uniformly 20 nm and the thicker shafts of the merozoite coat bristles appear to be the site of attachment to the erythrocyte surface. Short-range adhesion is also sometimes visible where other parts of the merozoite are attached to the erythrocyte and occasionally when merozoites adhere to each other.

Merozoites also sometimes adhere to neutrophil leucocytes by long- or short-range attachments. However, *P. knowlesi* merozoites incubated with mouse erythrocytes fail to form any adhesions of even a transitory nature (see also Butcher et al., *9*). These findings indicate that there are at least three distinct categories of receptor site on the merozoite cell coat, namely, those responsible for the cohesion of merozoite groups, those allowing loose, long-distance attachment to erythrocytes, and those mediating short-distance attachment at the merozoite apex, leading to invasion. The long extensible filaments of the cell coat appear to bear the first two of these receptor types whereas the thicker shafts, particularly in the apical region of the merozoite, bear the third class of receptor. The failure of *P. knowlesi* merozoites to adhere to mouse erythrocytes presumably implies that receptor–ligand interactions are of a specific chemical nature rather than being a nonspecific, perhaps electrostatic phenomenon.

Another intriguing aspect of adhesion is the spasmodic bending of erythrocytes that occurs immediately after merozoites have attached to them (*3*). Such movements may result from the progressive, zipper-like formation of tenuous connections between surfaces in the manner outlined above, although the relaxation of shape that follows a bending movement is difficult to explain; it is possible that attachments of this type are of limited duration, so that oscillations in the erythrocyte might be set up by the intermittent making and breaking of individual links with the attached merozoite. Alternatively, such changes in shape could be caused by the premature, diffusely acting release of membrane-active material from the rhoptries.

During invasion, the cell coat of merozoites is discarded and may be stripped from the parasite in some manner analogous to the " capping " of cell surface elements in other types of cell. The precise mechanics of this process, like much else concerning invasion, are obscure and need further investigation.

ENTRY INTO ERYTHROCYTES

Once the merozoite has attached by its apical prominence, the erythrocyte membrane expands inwards rapidly to form the rounded parasitophorous vacuole, sometimes accompanied by the formation of long, narrow channels reaching into the cytoplasm or of smaller subsidiary vacuoles (*2*). Since the apex of the merozoite is often at some distance from the lining of the major vacuole, it appears unlikely that this structure is formed by mechanical action, i.e., by pushing. Ladda et al. (*1*) noted that at the end of invasion the rhoptries appeared depleted of contents, and suggested that they could bring about vacuole formation by the release of a membrane-active substance from the apical complex of the parasite. In *P. knowlesi* we have found that, whilst the micronemes disappear early in invasion, the behaviour of the rhoptries is quite variable and they are often apparently intact until the initial formation of the vacuole is complete.

Studies on other sporozoa indicate that the rhoptries and micronemes form an interconnected system of channels. The rhoptries may therefore act as reservoirs into which the micronemes discharge before their contents are released from the cell, so that the micronemes would be the first to disappear. The final release of remaining material from the rhoptries is apparently associated mainly with the secondary expansion of the parasitophorous vacuole when the parasite begins to transform into a trophic organism, rather than with the initial creation of that cavity. Changes that can be seen in the rhoptries are reductions in their staining density and in their overall dimensions; they remain rounded however, indicating that although the rhoptry may discharge by diffusion of its concentrated contents, it may also contract actively.

Once the parasite is inside the erythrocyte, the remaining dense organelles (microspheres) are discharged by exocytosis through the pellicle and this process, together with the final secretion of the rhoptry contents, effects a rapid enlargement of the

parasitophorous vacuole, which becomes irregular in shape. In *P. knowlesi*, this process is complete within 5 min after contact is made with the erythrocyte (*2*). The cause of the various changes in the erythrocyte membrane is not yet understood, but the key to this problem must lie in the composition and properties of the rhoptries, micronemes, and microspheres (see the paper by A. Kilejian, pp. 191–197 of this issue).

Cytochemical studies recently carried out indicate that the rhoptries are largely proteinaceous, since they stain densely with ethanolic phosphotungstate and are not to any marked extent extractable with chloroform–methanol. The rhoptries discharge readily when merozoites are treated with ruthenium red either before or during fixation for electron microscopy (as also seen by Jensen & Hammond in *Eimeria*, *10*) to form lamellar bodies with an interperiod spacing of about 8 nm (Fig. 7). Such structures are presumably formed as complexes between the rhoptry contents and ruthenium red, since in sections and negatively stained preparations the intact rhoptries have a finely granular substructure. However, discharged rhoptry material, when negatively stained with phosphotungstate, also shows a lamellar configuration and membranous whorls reminiscent of such structures are present in the newly formed parasitophorous vacuoles in sections of specimens fixed with ordinary glutaraldehyde solutions (Fig. 8); this suggests that the contents of rhoptries can also form lamellar arrangements under more normal conditions.

These findings are in general agreement with those of Kilejian (*11*) who has correlated the presence of a histidine-rich protein with the rhoptry contents of *P. lophurae*; this protein is able to induce the invagination of avian erythrocyte membranes and is likely to be an agent of parasitophorous vacuole formation, although its mode of action is not clear.

Freeze fracture experiments reported by McLaren et al. (pp. 199–203 of this issue) indicate that the formation of the parasitophorous vacuole is initially caused by the inward buckling of the plasma membrane without any noticeable alteration in its composition, as determined from the density of membrane particles. We have also observed instances where merozoites have retained a zone of attachment between their surface and the wall of the vacuole, showing that at least that portion of the erythrocyte membrane had been invaginated intact. Recently, there has been considerable interest in the maintenance of cell shape in normal erythrocytes and in changes in form induced by various chemical treatments. Two alternative hypotheses are currently canvassed: one proposes that shape changes are caused by energy-dependent alterations in the proteins to which the erythrocyte membrane is attached (e.g., spectrin) (*12*); the other suggests that unequal expansion of the two lipid leaflets of the membrane bilayer could cause inward or outward bending (*13*, *14*). According to the latter view, amphiphilic, positively-charged molecules inserted into the inner membrane leaflet of the erythrocyte bring about its inward bending and thereby form endocytic vacuoles. In either case, changes in molecular configurations would not be readily detectable with the electron microscope; it can be calculated, for example, that a 1% local change in the area of the inner leaflet of the lipid bilayer would be enough to account for the formation of a vacuole 2 μm in diameter.

The drastic alterations in membrane structure seen in the later transformation of the vacuole show that the composition of the membrane must have been changed considerably (see also the paper by McLaren et al., pp. 199–203 of this issue) either by the removal of membrane proteins or by the addition of new materials to the membrane itself; whether the rhoptry contents are actually incorporated in the membrane is not known, although the obvious sudden expansion of the vacuole's surface area points to this conclusion.

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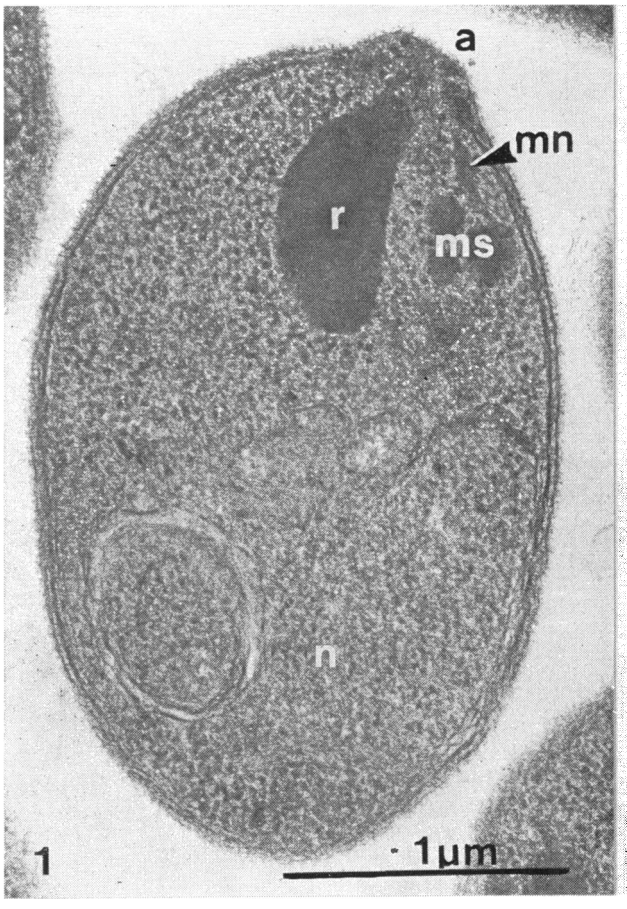
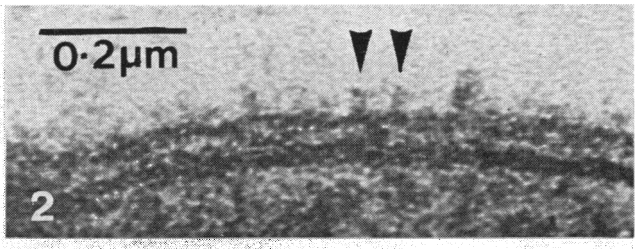
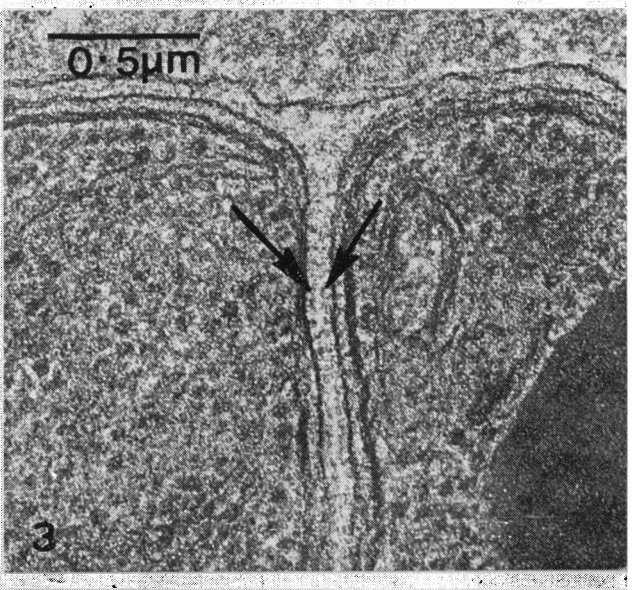






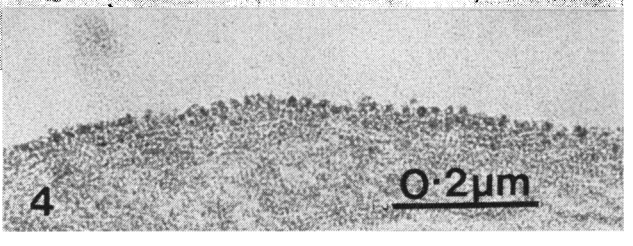
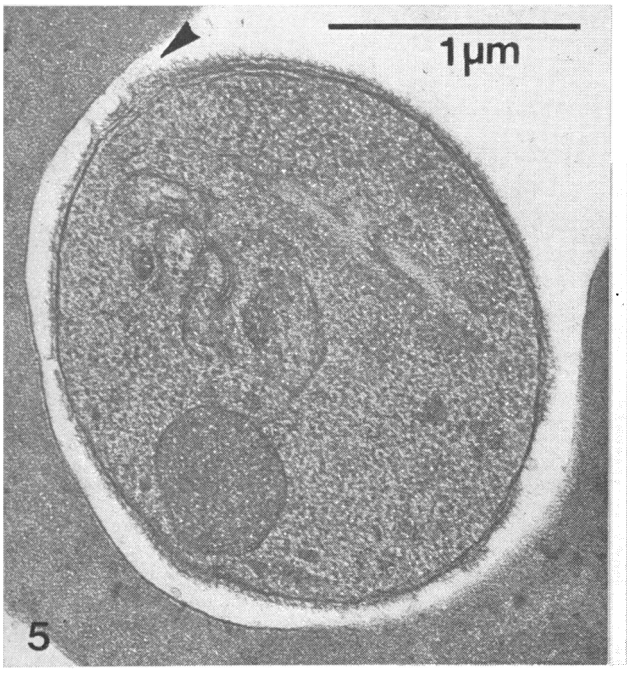


Fig. 1. Longitudinal section of an extracellular merozoite, showing its general structure including the apical prominence (a), microneme (mn), microspheres (ms), and nucleus (n). The cell exterior is bounded by a filamentous coat that has long thin connections with adjacent merozoites.

Fig. 2. A section through the pellicle of the merozoite stained for a long period in uranyl acetate to show the Y-shaped bristles (arrow heads).

Fig. 3. Portions of two adjacent merozoites within a schizont, showing regularly arranged lines of coat bristles attached to apposed surfaces (arrows).

Fig. 4. Cationized ferritin grains attached to the cell surface of an extracellular merozoite.



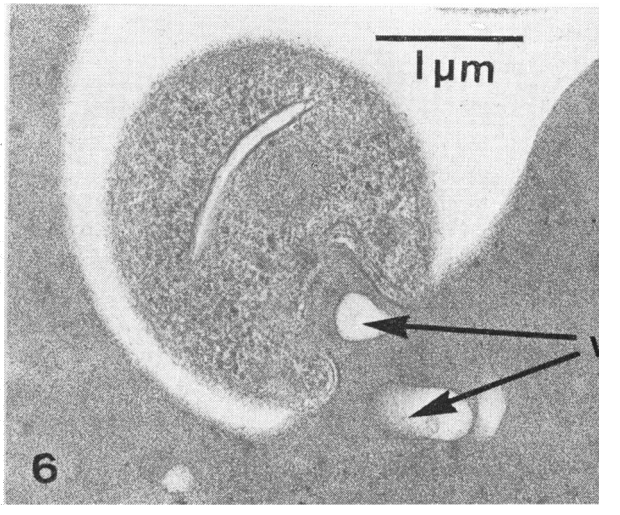
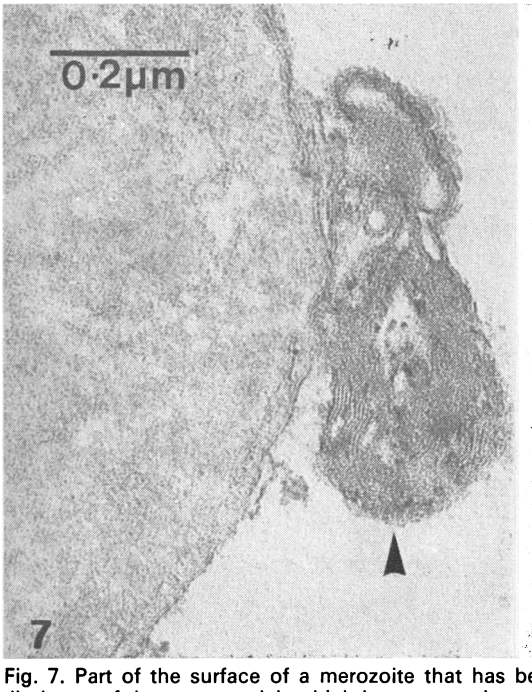


Fig. 5. A merozoite loosely attached to an erythrocyte by thin extensions of the parasite's coat (arrow head). Note the cup-like form of the erythrocyte, which is bent arround the merozoite in this type of adhesion.
Fig. 6. Contact between the apex of a merozoite and an erythrocyte. Note the manner in which the erythrocyte surface and the apical prominence of the merozoite are both greatly distorted, and the way in which membrane lined vesicles (v) appear within the erythrocyte.



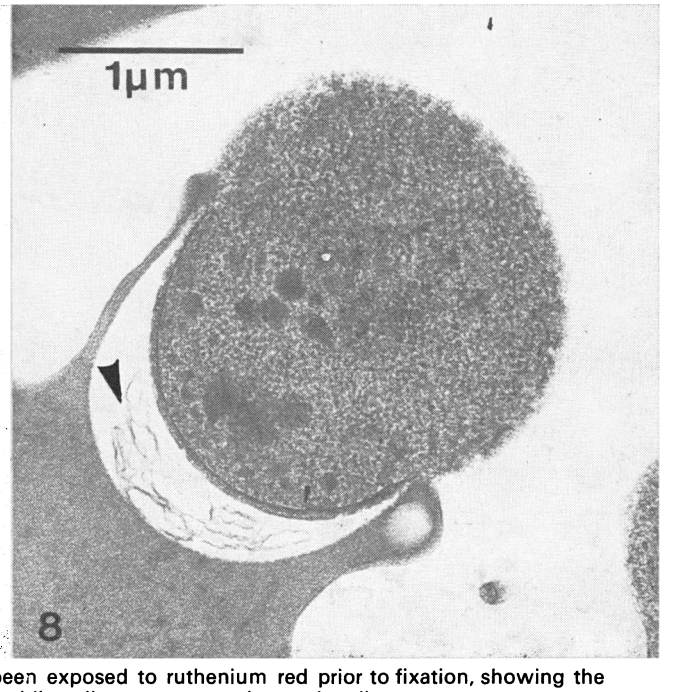


Fig. 7. Part of the surface of a merozoite that has been exposed to ruthenium red prior to fixation, showing the discharge of rhoptry material, which has assumed a multilamellar appearance (arrow head).
Fig. 8. Section through a merozoite invading a (crenated) erythrocyte, showing the formation of membranous lamellae in the forming parasitophorous vacuole (arrow head).

RÉSUMÉ

RÉCENTS PROGRÈS DANS LA CONNAISSANCE DU PHÉNOMÈNE DE PÉNÉTRATION DES MÉROZOÏTES DE *PLASMODIUM KNOWLESI* DANS LES ÉRYTHROCYTES

Les observations faites précédemment sur le processus de pénétration de *P. knowlesi* sont brièvement rappelées et de nouvelles constatations concernant l'adhérence des parasites aux érythrocytes et leur passage intracellulaire sont exposées. Les mérozoïtes adhèrent aux érythrocytes au moyen d'une enveloppe bien définie ayant les propriétés cytochimiques d'une glycoprotéine. Cette enveloppe possède des récepteurs qui interviennent dans au moins trois types distincts d'adhérence. L'invagination de la surface érythrocytaire qui en résulte comprend deux phases, la première consistant en un repli vers l'intérieur de la membrane qui forme ainsi une vacuole et la deuxième en une expansion rapide qui donne la vacuole parasitophore finale habitée par le parasite trophique. Les auteurs discutent enfin, en relation avec ces changements, de certaines constatations cytochimiques concernant la nature des structures touchant aux « rhoptries ».

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